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Synthesis and Evaluation of New Substrate Analogues of *Streptomyces* R61 DD-Peptidase: Dissection of a Specific Ligand

Rajesh Nagarajan and R. F. Pratt*

Department of Chemistry, Wesleyan University, Middletown, Connecticut 06459

rpratt@wesleyan.edu

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Good substrates of the *Streptomyces* R61 DD-peptidase, such as glycyl-L-α-amino-ϵ-pimelyl-D-alanyl-D-alanine, **1** (Anderson, J. W.; Pratt, R. F. *Biochemistry* **2000**, *39*, 12200−12209), contain the glycyl-L-α-amino-ϵ-pimelyl side chain. A number of thia variants of this structure have been synthesized by means of a disconnection strategy whereby the appropriate thiols were reacted with either acryloyl-D-alanyl-D-alanine or haloalkanoyl-D-alanyl-D-alanines. Kinetics studies of the hydrolysis of these compounds by the R61 DD-peptidase showed that the presence of the N-terminal glycylammonium ion and the pimelyl-α-carboxylate are very important for efficient catalysis. The results of deletion of the C-terminal D-alanine indicate a promising direction toward new inhibitors. Shorter (one methylene less) and longer (one methylene more) analogues of **1** are also poor substrates. Molecular modeling and dynamics studies suggest that the higher mobility of the active site residues and the modified substrates in enzyme−substrate complexes may be the dominant factor in this loss of reactivity. The general conclusion is that essentially all of the structural elements of the side chain of **1** are required to produce a good substrate. This result has important implications for the design of inhibitors of DD-peptidases.

Introduction

Bacterial transpeptidases or DD-peptidases are the targets of β-lactam antibiotics. Studies of the structure and function of these enzymes hold forth the promise of greater understanding of bacterial cell call assembly and new antibiotics. For many years, these studies were impeded by the small amount of material available from natural sources and the membrane-bound nature of the majority of these enzymes. More recently, however, advances in molecular biological techniques have led to the availability of pure water-solubilized versions of many DD-peptidases in quantities suitable for structural and chemical investigations. Indeed, there are now available X-ray crystal structures of several DD-peptidases.[1−5] Arguably not as well advanced, however, is knowledge of the DD-peptidases as catalysts.[6]

One would imagine, given the biological role of these enzymes,[7] that they would show substantial transpeptidase and carboxypeptidase activity against D-alanyl-D-alanine-terminating peptides, especially if further substituents were present causing the entire structure to resemble bacterial peptidoglycan. By and large, this expectation has not been convincingly demonstrated. Although modest activity against small peptides has been observed,[8−13] the measured rates, in general, would not be sufficient to keep a bacterium alive. Recently, in two instances, more active substrates have been discovered for particular DD-peptidases.[14,15] In this laboratory, we have shown that the peptide **1**, which mimics a portion of the peptidoglycan of *Streptomyces* sp., is an excellent substrate of the DD-peptidase of *Streptomyces* R61: k_{cat} = 69 s^{-1}, K_m= 7.9 μM, and k_{cat}/K_m= 8.7 × 10^6 s^{-1} M^{-1}.[14] An X-ray crystal structure of this peptide bound to the *Streptomyces* R61 DD-peptidase revealed a specific binding site for the α-glycyl-ϵ-pimelyl side chain.[16] Less specific side chains produced considerably poorer substrates, e.g., k_{cat}/K_m values for **2** and **3** are 3.5 × 10^3 s^{-1} M^{-1} [17] and 286 $s^{-1}M^{-1}$,[18] respectively.

* Address correspondence to this author. Phone: 860-685-2629. Fax: 860-685-2211.

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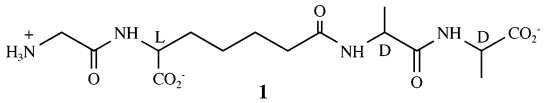

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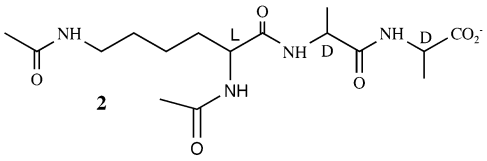

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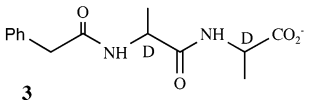

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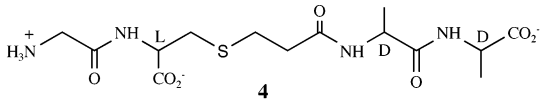
In view of the disparate results obtained with various peptides, as indicated above, it seemed useful to examine the specificity of **1** in more detail. The specificity with respect to the C-terminal amino acids has been previously studied.[19] We were interested, therefore, in finding how much the various structural elements of the side chain of **1** contributed to catalysis. To do this, a number of variant structures of **1** were required. Since the synthesis of **1** is tedious, given that L-α-aminopimelic acid is not commercially available, and requires a number of protection and deprotection steps, we decided to explore the disconnection strategies illustrated in Scheme 1. It was anticipated that these procedures would be versatile and require many fewer steps than the original synthesis of **1**.

In this paper we describe the synthesis of a number of variants of **1**, viz. **4**−**8**, and examine their specificity for the *Streptomyces* R61 DD-peptidase. We find that the enzyme active site is extremely specific for the side chain of **1** in its entirety.



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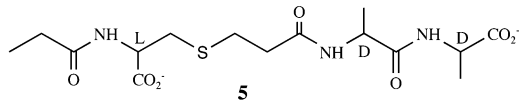

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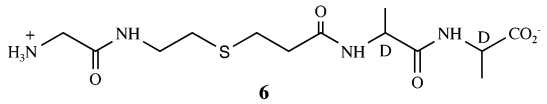

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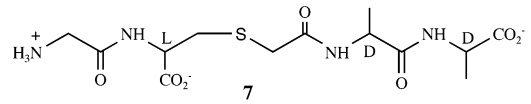

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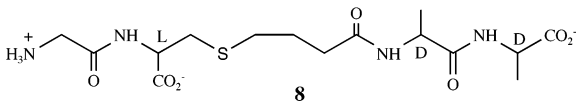

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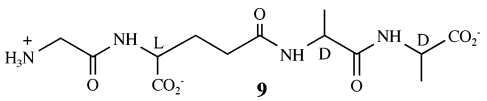
Results and Discussion

The synthesis of **4**−**6** proceeded smoothly according to Scheme 1a where the thiols employed were glycyl-L-cysteine, *N*-acetyl-L-cysteine, and glycylcysteamine, respectively. The shorter and longer side chain variants, **7** and **8**, respectively, were obtained by the reactions of Scheme 1b. Compound **7** was obtained from reaction of glycyl-L-cysteine with *N*-(iodoacetyl)-D-alanyl-D-alanine and **8** from reaction of the same thiol with *N*-(4-bromobutyryl)-D-alanyl-D-alanine. All compounds required chromatographic purification to remove small amounts of starting materials, the disulfides from thiol oxidation, iodoacetate from hydrolysis of iodoacetyl chloride in the synthesis of **7**, and the intramolecular cyclization side reaction product from 4-(*N*-bromobutyryl)-D-alanyl-D-alanine[20] in the synthesis of **8**.

NMR experiments showed that the R61 DD-peptidase catalyzed the hydrolysis of **4**−**8** (Scheme 2; L and P represent D-alanine and the carboxylate products, respectively); in each case the distinctive resonances of D-alanine at 1.5 and 3.8 ppm increased with time after addition of the enzyme until the reaction was complete. Even in these qualitative experiments, it was obvious that **4** was a much better substrate than **5**−**8**.

Steady-state kinetic parameters for the hydrolysis of **4**−**8** by the peptidase were determined as described in the Experimental Section and are listed in Table 1. Also presented in Table 1 are comparable values of these parameters for **1** and nonspecific peptides **2**, **3**, and **9**.



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An immediate indication of the high structural specificity of **1** is seen in the comparison of its kinetics parameters with those of **4**, where the only difference is the replacement of $-CH_2-$ by $-S-$ in the tetramethylene chain. This change would bring about small changes in geometry. The C−S bond is longer than the C−C bond (1.82 and 1.53 Å, respectively) but this difference is offset by the more acute C−S−C angle than that of C−C−C (ca. 100° vs 110°). Energy-minimized free peptide models in extended conformations yielded distances of 6.55 and 6.77 Å for the aliphatic chains (ϵ-carbonyl carbon to α-carbon) of **1** and **4**, respectively. Although the slightly different geometry of the C−S−C moiety might prove significant in a restricted space situation, it is not clear that this is one (see below).

More responsible, probably, for the difference in steady-state kinetics between **1** and **4** when hydrolyzed in the presence of the R61 DD-peptidase is the difference in hydrophobicity between the side chains. Values of log *P* for pentane and diethyl disulfide are 3.39 and 1.95, respectively.[21] This difference corresponds to a difference of 2 kcal/mol in the free energy of transfer from water into *n*-octanol in favor of the hydrocarbon. The difference in free energy of activation of **1** and **4** in the enzyme-

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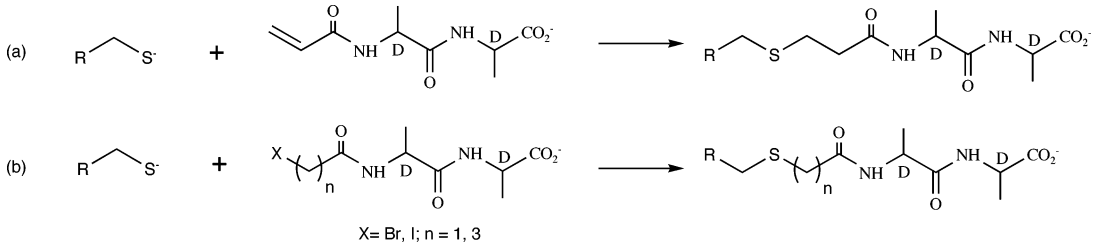
SCHEME 1



SCHEME 2

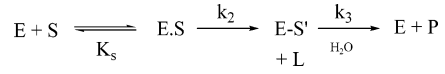


TABLE 1. Steady State Kinetic Parameters for Peptide Hydrolysis by the *Streptomyces* R61 DD-Peptidase

peptide	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (s^{-1} M^{-1})
1[a]	69	7.9	8.7×10^6
4	48 ± 8	260 ± 100	1.85×10^5
5	0.25 ± 0.01	1280 ± 90	197
6	≥2.4	≥3000	790
7	0.17 ± 0.01	860 ± 170	200
8	0.27 ± 0.01	480 ± 160	560
2[b]	34.5	9.8×10^3	3.52×10^3
3[c]	2	7×10^3	280
9[d]	12.8	860	1.5×10^4

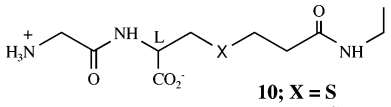
[a] Data from ref 14. [b] Data from ref 17. [c] Data from ref 18. [d] Data from ref 14.

catalyzed hydrolysis (k_{cat}/K_m) is 2.3 kcal/mol, also in favor of the hydrocarbon. The crystal structure of **1** bound to the enzyme shows the tetramethylene side chain bound in a hydrophobic region adjacent to the side chains of Phe 120 and Trp 223. It is likely then that the difference in the rates of hydrolysis of **1** and **4**, as illustrated by the k_{cat}/K_m values of Table 1, arises largely from this hydrophobicity factor. The compounds **5**−**8** also have thia-substituted side chains and thus the kinetics results from these compounds (Table 1) should be compared with those from **4**.

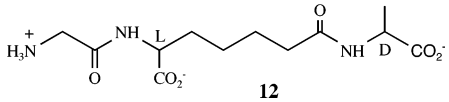
Compound **5** is significantly poorer as a substrate than **4**. Apparently the terminal amine, most likely in protonated form, is important for catalysis. In this case, the k_{cat} parameter seems most affected. It is likely, by analogy to the situation with **1**[14] and other peptide substrates of the R61 DD-peptidase, that k_{cat} corresponds to k_2, the acylation rate constant (Scheme 2, where E.S represents the noncovalent complex of enzyme and substrate and E-S′ the acyl-enzyme intermediate formed from enzyme acylation and release of the leaving group L). In the case of **5**, therefore, noncovalent association is not strongly affected but the complex formed is not nearly as productive as the analogous complex formed from **4**. This suggests that binding of the polar terminus of the side chain of **1** may be involved in an induced fit type activation of the active site.

Compound **6** is also a much poorer substrate than **4**. Deletion of the adipyl α-carboxylate clearly has negative effects on catalysis. In this case, however, there is a more significant effect on noncovalent binding (K_s) than occurs with **5**. The acylation rate may also be affected.

The contribution of the C-terminal D-alanine to binding was estimated by measurement of the inhibitory power of **10**. This compound, prepared by addition of glycyl-L-cysteine to *N*-ethylacrylamide, as in Scheme 1a, contains the elements of the side chain and the penultimate D-alanine of **4**, but lacks the C-terminal D-alanine. The K_i value of **10** as an inhibitor of the R61 DD-peptidase



10; X = S
11; X = CH_2



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was measured against turnover of the substrates *m*-{[*N*-phenylacetyl]oxy}benzoic acid and *N*,*N*′-diacetyl-L-lysyl-D-alanyl-D-alanine and against inhibition of the enzyme by dansylpenicillin.[14] The results of these experiments were the same: the K_i value of **10** was 1.02 ± 0.12 mM. Loss of the terminal D-alanine from **4** thus increases the dissociation constant by some 4-fold; on the basis of the difference in k_{cat}/K_m values between **1** and **4**, the carbon chain analogue of **10**, viz. **11**, may thus have a dissociation constant of around 30 μM. It might also be noted here that **12**, the hydrolysis product of **1**, has a K_i value of 160 μM against **1**;[14] the C-terminal carboxylate of **12** is known, however, to disrupt the active site.[16]

The importance of the length of the side chain on catalysis was explored with compounds **6** (shorter than **4**) and **7** (longer). Energy-minimized aliphatic chain lengths (ϵ-carbonyl carbon to the α-carbon of the pimelyl residue) from models of **4**, **6**, and **7** were 6.77, 5.21, and 7.43 Å, respectively. These changes in geometry have had dramatic negative effects on catalysis, where both k_2 and K_s have been significantly affected. The effects brought about by these changes have been as drastic as those produced by removal of the functional groups described above. The complete structure of **1** is therefore very important for optimal catalysis.

The change in catalytic parameters can now be considered in terms of structure. Structures of a noncovalent complex of **1** with the R61 DD-peptidase and of a tetrahedral phosphonate transition state analogue of **1** are available.[16,22] The glycyl-α-L-amino-ϵ-pimelyl side chain interacts strongly with the enzyme in an identical fashion in these two structures. The ammonium terminus is within hydrogen-bonding distances of the backbone carbonyl oxygen of Phe 120 and the side chain hydroxyl group of Thr 123. The α-carboxylate appears to be

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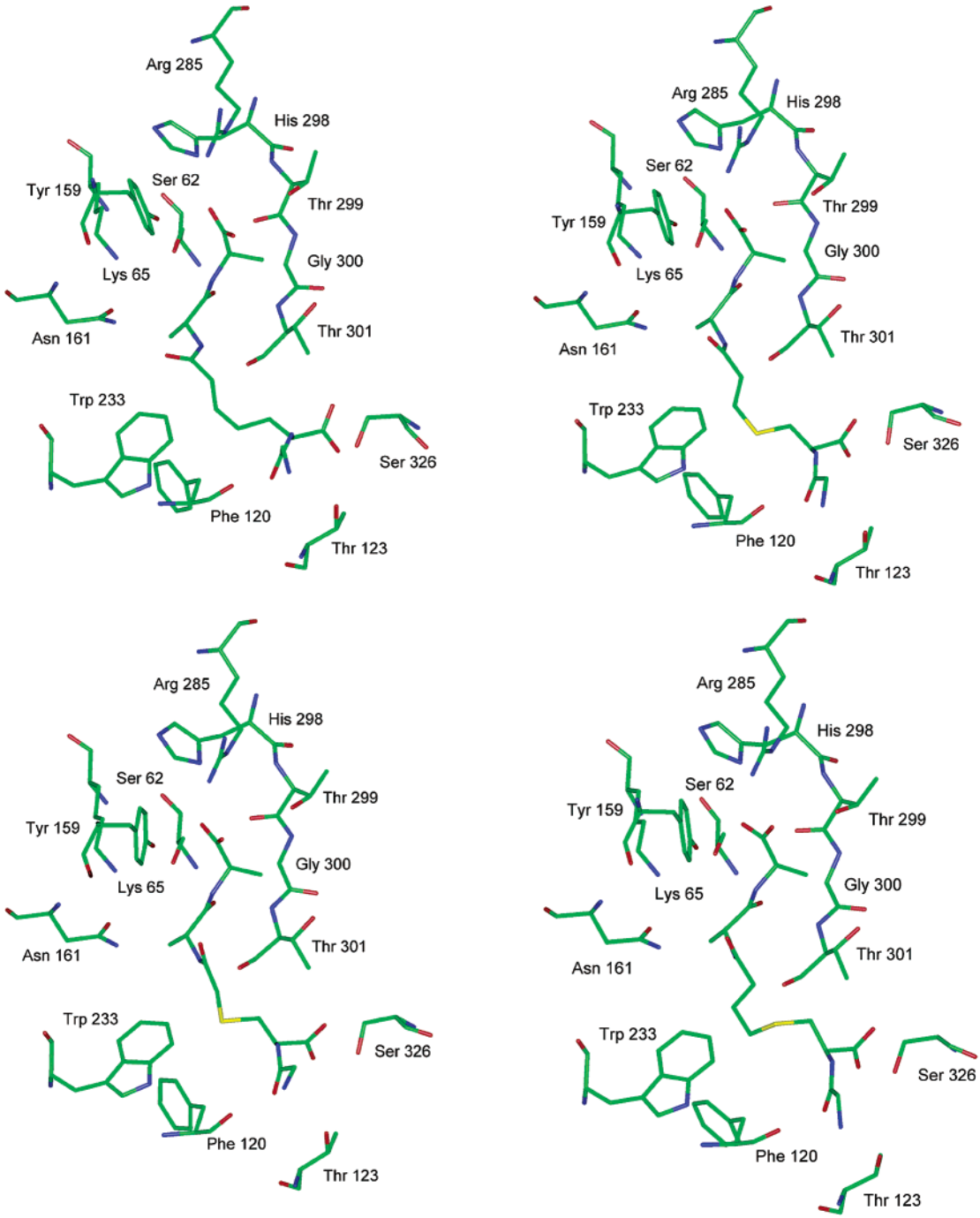

FIGURE 1. Energy-minimized structures of **1** (upper left), **4** (upper right), **7** (lower left), and **8** (lower right) at the active site of the *Streptomyces* R61 DD-peptidase.

hydrogen bonded to the backbone NH of Asn 327 and the side chain hydroxyl group of Ser 326. Finally, the tetramethylene linker lies in close contact (3.4−3.6 Å) with the hydrophobic side chains of Phe 120 and Trp 233. The results of the experiments described above show that each of these interactions is important for catalysis. The loss of any one of them must lead to an enzyme less coupled to a more mobile substrate and the decrease in such coupling must directly affect catalysis.

The reason(s) for the much-reduced activity of the shorter and longer substrates, **7** and **8**, respectively, was not immediately obvious. Modeling showed that it is possible with both of them to obtain energy-minimized structures of both noncovalent and covalent (tetrahedral intermediate) complexes where they appear to interact with the active site as well as do **1** and **4**, substrates of presumably close to optimal length. The shorter substrate, **7**, is able to extend to span the binding site while the longer, **8**, is able to fold in such a way as to do likewise. These points can be seen in Figure 1 where these energy-minimized noncovalent structures are shown.

Differences did emerge, however, when the results of the molecular dynamics computations were considered. From the 200-ps runs of the noncovalent complexes, 2-ps snapshots from 50 to 200 ps were averaged and the

TABLE 2. Mobility of the Active Site and Substrate in Complexes of Peptides 1, 4, 7, 8, and 2 with the R61 DD-Peptidase

peptide	rms distance (Å)[a] active site plus ligand	rms distance (Å)[a] ligand alone
1	0.73	0.78
4	0.92	0.88
7	0.98	1.45
8	1.24	1.49
2	1.08	0.97

[a] Distances between active site components of time-averaged structures and corresponding energy minimized structures (see text for details).

averaged structures compared with the initial energy-minimized structure in each case. The latter was assumed to represent the catalytically active form because of the distribution of functional groups (see above). The root-mean-square differences between the dynamic structure and the initial structure in each case are reported in Table 2. These differences were obtained from overlays of the residues of the active site shown in Figure 1 and the ligand from the averaged structure onto the initial structure by means of ProFit.[23] It is clear from Table 2 that the active site region, including substrate, is most mobile in the case of the extended substrate, **8**, less so in that of the shorter substrate, **7**, and least so in that of the original specific substrate **1**.

Distinctively more mobile residues in the complexes of **4**, **7**, and **8** with respect to that of **1** were Tyr159, Asn161, Gly300, Thr301, and Ser326. Differences that would likely be directly related to catalysis include a loosening of the oxyanion hole interactions in complexes of **4**, **7**, and **8** (average values of the $O^{-}\cdots$HNThr301 distance over the 150 ps interval described above were 2.95 ± 0.13, 3.14 ± 0.24, 3.05 ± 0.20, and 3.85 ± 0.80 Å, respectively, for **1**, **4**, **7**, and **8**). Also likely to be significant were changes in the Ser62 O$\gamma\cdots$C=O (substrate) distance (3.15 ± 0.21, 4.04 ± 0.25, 3.39 ± 0.22, and 4.73 ± 0.30 Å, respectively) and the Tyr159 O$\cdots$Ser62 Oγ distance (2.72 ± 0.21, 4.26 ± 0.32, 4.01 ± 0.78, and 4.17 ± 0.34 Å, respectively). Tyr150, in the anionic form, may be the general base involved in catalysis[22,24] and, in crystal structures, is generally closely associated with Ser62 Oγ, the active site nucleophile. The movement of Tyr159 away from Ser62 in the dynamic complexes of the enzyme with **4**, **7**, and **8** would therefore be in accord with the looser association of these substrates with the active site and thus less effective catalysis than observed with **1**.

Our modeling results with the substrate **2** are also of interest, first because of the high k_{cat} value for this substrate (Table 1) despite its very high K_m, and because it has been used as a generic small molecule substrate of DD-peptidases for many years.[19] The dynamic model of **2**, noncovalently bound to the active site of the R61 DD-peptidase and constructed as for the other substrates, showed that the active site and ligand are more mobile with **2** than with **1** and **4**, but less so than with **7** and **8** (Table 2). This result supports the general correlation deduced above between active site mobility and catalysis (k_{cat}/K_m values; Table 1). Notably, the model of **2** with the enzyme suggests that neither of the acetamido groups of **2** interact directly with any specific components of the active site. This would account for the apparently weak overall binding (high K_m). On the other hand, hydrophobic interaction of the tetramethylene side chain with Trp 233 and Phe 120 must be sufficiently effective to induce a quite reactive conformation of the active site for catalysis (high k_{cat}).

In conclusion, we have shown that a disconnection synthetic strategy is a promising avenue of approach to α-aminopimelyl analogues. Facile synthesis of **4**−**8** has thus been achieved. Extension of the strategy to diaminopimelates is possible. The peptides thus prepared were investigated as substrates of the *Streptomyces* R61 DD-peptidase. The results obtained with **5** and **6** demonstrate that both the terminal amine (ammonium ion) and the pimelyl carboxylate are important for effective catalysis. The rather surprising results with **7** and **8**, the shorter and longer analogues of **4**, further demonstrate that a precise fit of the polar glycyl-α-aminopimelyl terminus of the side chain into its binding site is required for efficient catalysis. It is likely that this result is general for DD-peptidases, i.e., that precise occupancy of some part at least of the extended substate binding site is needed for significant DD-peptidase activity. The results from the molecular dynamics simulations show that loss of even part of the structural elements of **1** leads to a more mobile active site and bound substrate. This increased mobility must presumably be entropically detrimental to catalysis. These issues require further study but should lead to new leads for DD-peptidase inhibitors and, possibly, antibiotics.

Experimental Section

Synthesis: 3-(*N*-Glycyl-L-cysteinyl)propanoyl-D-alanyl-D-alanine (4). *N*-Acryloyl-D-alanyl-D-alanine was prepared in 52% yield as described by Hiese et al.[25] ^{1}H NMR (D_2O) δ 1.41 (3H, d, $J = 7$ Hz), 1.44 (3H, d, $J = 7$ Hz), 4.38 (1H, quartet, $J = 7.2$ Hz), 4.35 (1H, quartet, $J = 7.8$ Hz), 5.8 (1H, dd, $J = 3$, 9 Hz), 6.2 (1H, dd, $J = 3$, 18 Hz), 6.3 (1H, dd, $J = 9$, 18 Hz).

Michael addition of *N*-glycyl-L-cysteine to *N*-acryloyl-D-alanyl-D-alanine was achieved by the general procedure of Friedman et al.[26] *N*-Glycyl-L-cysteine (167 mg, 93 mmol) was dissolved in 2 mL of water and the pH of the solution was raised to 9.5 by addition of ammonium hydroxide. *N*-Acryloyl-D-alanyl-D-alanine (200 mg, 93 mmol) was added to the reaction mixture, which was stirred under nitrogen for 48 h. The pH of the solution was maintained at 9.5 throughout the reaction period by addition of ammonium hydroxide. After the reaction was complete (48 h; checked by NMR), the reaction mixture was freeze-dried and the product purified as the ammonium salt by Biogel P2 chromatography with water as the eluent (55%). ^{1}H NMR (D_2O) δ 1.33 (3H, d, $J = 7$ Hz), 1.37 (3H, d, $J = 7$ Hz), 2.59 (2H, t, $J = 9$ Hz), 2.83 (2H, t, $J = 9$ Hz), 2.90 (1H, dd, $J = 9$, 14 Hz), 3.10 (1H, dd, $J = 4.5$, 12 Hz), 3.82 (2H, s), 4.11 (1H, quartet, $J = 7.5$ Hz), 4.32 (1H, quartet, $J = 7.5$ Hz), 4.40 (1H, dd, $J = 3$, 6 Hz); exact mass (ES^+) MH^+ calcd for $C_{14}H_{25}N_4O_7S$ 393.1444, found 393.1444.

3-(*N*-Acetyl-L-cysteinyl)propanoyl-D-alanyl-D-alanine (5). This material was prepared by Michael addition of

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N-acetyl-L-cysteine to *N*-acryloyl-D-alanyl-D-alanine in exactly the same way as for **4**. The product was purified as the ammonium salt by means of Sephadex G10 chromatography (38%). ^{1}H NMR (D_2O) δ 1.43 (3H, d, J = 7 Hz), 1.47 (3H, d, J = 7 Hz), 2.2 (3H, s), 2.59 (2H, t, J = 9 Hz), 2.82 (2H, t, J = 9 Hz), 2.95 (1H, dd, J = 9, 14 Hz), 3.05 (1H, dd, J = 4.5, 12 Hz), 4.12 (1H, quartet, J = 7.5 Hz), 4.32 (1H, quartet, J = 7.5 Hz), 4.33 (1H, dd, J = 3, 6 Hz); exact mass (ES^+) MH^+ calcd for $C_{14}H_{24}N_3O_7S$ 378.1335, found 378.1350.

3-(*N*-Glycyl-cysteaminyl)propanoyl-D-alanyl-D-alanine (6). *N*-Boc-glycyl-cystamine was prepared from the reaction of cystamine hydrochloride with Boc-glycine hydroxysuccinimide ester, followed by deprotection and reduction to *N*-glycyl-cysteamine, as described by Shair et al.[27] Cystamine hydrochloride (0.918 g, 8.1 mmol) and $NaHCO_3$ (1.23 g, 14.6 mmol) were dissolved in 100 mL of water and Boc-glycine hydroxysuccinimide ester (2 g, 7.34 mmol), dissolved in 100 mL of dichloromethane, was added. The reaction mixture was stirred at room temperature for 3 h, and then extracted with dichloromethane (twice). The combined organic layers were washed with 5% aq HCl, 10% $NaHCO_3$ solution, and finally with water, and dried over $MgSO_4$. The solvent was then evaporated and the product dried with a vacuum pump (37%). ^{1}H NMR ($CDCl_3$) δ 1.43 (9H, s), 2.60 (2H, quartet, J = 8 Hz), 3.42 (2H, quartet, J = 8 Hz), 3.78 (2H, d, J = 8 Hz).

N-Boc-glycyl-cystamine (700 mg, 3 mmol) was dissolved in 15 mL of methylene chloride. The reaction vessel was flushed with argon. Triethylsilane (2.63 mL, 16 mmol) and 1.3 mL of trifluoroacetic acid were then added to the vessel and the reaction mixture was stirred at room temperature for 90 min. The reaction mixture was then concentrated and dried with a vacuum pump for 30 min, yielding the product, glycyl-cysteamine. ^{1}H NMR (D_2O) δ 2.68 (2H, t, J = 8 Hz), 3.44 (2H, t, J = 8 Hz), 3.82 (2H, s).

The crude glycyl-cysteamine was then reacted with *N*-acryloyl-D-alanyl-D-alanine as described for **4**. After the reaction was complete (48 h; confirmed by NMR), the reaction mixture was freeze-dried and purified by passage through a Biogel P2 column with water as the eluent (46%). ^{1}H NMR (D_2O) δ 1.34 (3H, d, J = 7 Hz), 1.38 (3H, d, J = 7 Hz), 2.59 (2H, t, J = 8 Hz), 2.74 (2H, t, J = 8 Hz), 2.83 (2H, t, J = 8 Hz), 3.48 (2H, t, J = 8 Hz), 3.78 (2H, s), 4.11 (1H, quartet, J = 7.5 Hz), 4.32 (1H, quartet, J = 7.5 Hz); exact mass (ES^+) MH^+ calcd for $C_{13}H_{25}N_4O_5S$ 349.1546, found 349.1541.

2-(*N*-Glycyl-L-cysteinyl)acetyl-D-alanyl-D-alanine (7). *N*-Iodoacetyl-D-alanyl-D-alanine was first prepared from the reaction of D-alanyl-D-alanine with iodoacetyl chloride. Thus, D-alanyl-D-alanine (3 g, 19 mmol) was dissolved in 15 mL of water and 1 M Na_2CO_3 was added dropwise to bring the pH to 8.5. Iodoacetyl chloride (3.84 g, 25 mmol) was added dropwise to the stirred reaction mixture in the cold room over a period of 1 h. After the reaction was complete (1.5 h; confirmed by NMR), the reaction mixture was freeze-dried. An NMR spectrum of the product mixture showed that it contained the product (80%), iodoacetate (10%), and a comparable amount of unreacted D-ala-D-ala. This mixture was used for coupling with glycyl-L-cysteine. ^{1}H NMR (D_2O) δ 1.34 (3H, d, J = 7 Hz), 1.39 (3H, d, J = 7 Hz), 3.80 (2H, s), 4.11 (1H, quartet, J = 7.5 Hz), 4.29 (1H, quartet, J = 7.5 Hz).

N-Iodoacetyl-D-alanyl-D-alanine (300 mg, 0.92 mmol) was dissolved in 1 mL of water and the pH of the solution was brought to 8.5 with 1 M Na_2CO_3. Glycyl-L-cysteine (150 mg, 0.84 mmol) was dissolved in 500 μL of water and the pH was brought to 8.5. This solution was added to the reaction mixture containing *N*-iodoacetyl-D-ala-D-ala at room temperature. The reaction mixture was then stirred for 7 h under nitrogen and freeze-dried. The product mixture was purified with Biogel P2 chromatography with water as the eluent (38%). ^{1}H NMR (D_2O) δ 1.33 (3H, d, J = 7 Hz), 1.39 (3H, d, J = 7 Hz), 2.94 (1H, dd, J = 9, 14 Hz), 3.03 (1H, dd, J = 4.5, 12 Hz), 3.33 (2H, s), 3.74 (2H, s), 4.12 (1H, quartet, J = 7.5 Hz), 4.31 (1H, quartet, J = 7.5 Hz), 4.41 (1H, dd, J = 3, 6 Hz); exact mass (ES^+) MH^+ calcd for $C_{13}H_{23}N_4O_7S$ 379.1287, found 379.1289.

3-(*N*-Glycyl-L-cysteinyl)butanoyl-D-alanyl-D-alanine (8). 4-Bromobutyryl-D-alanyl-D-alanine was prepared following the general procedure of Reid et al.[28] D-Alanyl-D-alanine (172 mg, 1.1 mmol) was dissolved in 3 mL of THF and 3 mL of water. To this solution at room temperature were added 4-bromobutyryl chloride (150 μL, 1.21 mmol), dissolved in 500 μL of THF, and 20% aqueous K_2CO_3 in alternate portions and the mixture was stirred for 30 min. The reaction was then complete (confirmed by NMR) so the THF was evaporated and the reaction mixture was freeze-dried. Purification of 4-bromobutyryl-D-alanyl-D-alanine [from a small amount of the cyclized material[20]] was not attempted at this stage because the glycyl-L-cysteine substitution in the next step required base, which would probably induce more cyclization. ^{1}H NMR (D_2O) δ 1.33 (3H, d, J = 7 Hz), 1.37 (3H, d, J = 7 Hz), 2.13 (2H, m), 2.55 (2H, t, J = 7.5 Hz), 3.50 (2H, t, J = 7.5 Hz), 4.12 (1H, quartet, J = 7.5 Hz), 4.30 (1H, quartet, J = 7.5 Hz).

The reaction of the 4-bromobutyryl-D-alanyl-D-alanine with *N*-gly-L-cysteine was carried out by a procedure adapted from Gibb et al.[29] Glycyl-L-cysteine (200 mg, 1.1 mmol) was dissolved in 7 mL of DMF, followed by the addition of 185 μL of DBU and 4-bromobutyryl-D-alanyl-D-alanine (400 mg, 1.1 mmol). The reaction mixture was stirred under nitrogen at room temperature for 44 h, DMF was removed by evaporation under vacuum, water (1 mL) was added, and the mixture was freeze-dried. The crude product was purified with Biogel P2 chromatography, followed by LH20 chromatography, and eluted with water in both cases (21%). ^{1}H NMR (D_2O) δ 1.33 (3H, d, J = 7 Hz), 1.37 (3H, d, J = 7 Hz), 1.88 (2H, m), 2.38 (2H, t, J = 7.5 Hz), 2.59 (2H, t, J = 7.5 Hz), 2.96 (1H, dd, J = 9, 14 Hz), 3.04 (1H, dd, J = 4.5, 12 Hz), 3.58 (2H, s), 4.11 (1H, quartet, J = 7.5 Hz), 4.29 (1H, quartet, J = 7.5 Hz), 4.38 (1H, dd, J = 3, 6 Hz); exact mass (ES^+) MH^+ calcd for $C_{15}H_{27}N_4O_7S$ 407.1600, found 407.1600.

3-(*N*-Glycyl-L-cysteinyl)-*N*-ethylpropionamide (10). Glycyl-L-cysteine (60 mg, 0.33 mmol) was dissolved in the minimum amount of water. The pH of the solution was brought to 8.5 with ammonium hydroxide. *N*-Ethylacrylamide (33 mg, 0.33 mmol) was then added to the reaction mixture. The system was flushed with nitrogen and the pH was maintained at 8.5 throughout the reaction period. After 48 h, the reaction mixture was freeze-dried and the product purified by Biogel P2 chromatography (32%). ^{1}H NMR (D_2O) δ 1.10 (3H, t, J = 8 Hz), 2.52 (2H, t, J = 9 Hz), 2.82 (2H, t, J = 9 Hz), 2.92 (1H, dd, J = 9, 14 Hz), 3.08 (1H, dd, J = 4.5, 12 Hz), 3.20 (2H, quartet, J = 8 Hz), 3.77 (2H, s), 4.41 (1H, dd, J = 3, 6 Hz); exact mass (ES^+) MH^+ calcd for $C_{10}H_{20}N_3O_4S$ 278.1175, found 278.1170.

Kinetics. A general NMR experiment was first used to demonstrate that **4**−**8** were in fact substrates of the *Streptomyces* R61 DD-peptidase. NMR spectra were recorded of solutions of the compound (2−4 mM) in D_2O/sodium bicarbonate (20−30 mM) before and after addition of the enzyme (final concentration 27 nM). Further spectra were taken as a function of time until the reaction was complete.

All quantitative kinetics experiments were carried out in 10 mM phosphate buffer at pH 7.5 and 25 °C. Spectrophotometric initial rates of turnover of **4** were measured at 220 nm. The enzyme concentration was 32 nM and substrate concentrations were varied from 50 to 800 μM. The initial rates were fitted to the Michaelis−Menten equation by means of a nonlinear least-squares computer program.

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Values of K_m for the poorer substrates **5**−**8** were obtained as inhibition constants, K_i, from inhibition of turnover of the depsipeptide substrate *m*-{[*N*-(phenylacetyl)alanyl]oxy}benzoic acid.[30] The substrate concentration was 1.0 mM in all experiments and initial rates were determined spectrophotometrically at 290 nm. Concentrations of **5** and enzyme were 0.5−3.0 mM and 60 nM, respectively, of **6** and enzyme 0.5−3.0 mM and 32 nM, respectively, of **7** and enzyme 0.1−1.5 mM and 60 nM, respectively, and of **8** and enzyme 0.5−1.0 mM and 85 nM, respectively. In each case, the initial rates were plotted versus inhibitor (peptide) concentration and fitted to a simple competitive inhibition equation by a nonlinear least-squares procedure to obtain a K_i value; the K_m value of the depsipeptide substrate was taken as 1.0 mM.[30] Total progress curves for direct turnover of **5**−**8** (**5**, 0.5, 0.75, 1.0 mM; **6**, 0.5, 1 mM; **7**, 0.5 mM; **8**, 0.5, 1.0 mM) by the dd-peptidase (0.32 μM) were obtained spectrophotometrically at either 220 or 225 nm. These were fitted to a Michaelis−Menten scheme to obtain k_{cat} values by means of the Dynafit program;[31] K_m values were fixed at the K_i values obtained as described above.

Molecular Modeling. The computations were performed by means of an sgi octane2 workstation with the InsightII 2000 program (MSI, San Diego, CA). The starting point for the structures was the crystal structure of the R61 DD-peptidase with a phosphonate inhibitor bound at the active site and including the crystallographic water molecules.[22] A tetrahedral intermediate structure derived from **1**, covalently bound to the active site serine nucleophile, was built onto this structure by means of the Builder module of InsightII.[32] The AMPAC/MOPAC module was used to calculate MNDO charges for the ligand. The charges on the protein were assigned by the Builder module in InsightII. The ligand was initially oriented with the oxyanion in the oxyanion hole, and the pimelyl side chain was oriented in the same position as seen in the crystal structure (phosphorus in the phosphonate inhibitor was changed to carbon). The tetrahedral intermediate from **4** was built from the R61-**1** complex by converting the appropriate methylene in the pimelyl side chain to sulfur. Intermediates from **7** and **8** were built from the R61-**4** complex. A 20-Å sphere of water molecules centered at Ser62 Oγ was added to each structure. The side chains of Lys65 and Tyr159 were cationic (protonated) and neutral, respectively. The ligand had formal charges on the tetrahedral oxyanion (O^-), the terminal amine (NH_3^+), and the carboxylate groups of the pimelyl side chain and the D-alanine leaving group.

The enzyme−ligand complexes were subjected to an energy-minimization procedure of 1000 steps of the steepest descent method followed by 2000 steps of conjugate gradients with use of the Discover program (version 2.98). The energy-reduced structures were then employed as the starting structures for molecular dynamics simulations (200 ps). A CV force field was employed for both the molecular mechanics and dynamics.

To build the noncovalent ligand complexes with the DD-peptidase, the energy-minimized tetrahedral intermediates were converted to noncovalent structures by breaking the covalent bond between C1 and Ser62 Oγ, the oxyanions were converted to carbonyls, and hydrogens were attached to Oγ in Ser62 with use of the Builder module in InsightII. MNDO charges calculated with the AMPAC/MOPAC module were assigned to the ligand. A fresh 20-Å sphere of water molecules was added to each structure centered at Ser62 Oγ. In each case, the complex was first energy-minimized as described above. This minimized structure was then used as the starting structure for 200 ps dynamics runs. Root-mean-square differences between structures were determined by the program ProFit.[23]

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Supporting Information Available: Materials and methods and proton NMR spectra of all new compounds and important intermediates. This material is available free of charge via the Internet at http://pubs.acs.org.

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